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Photochemical Cross-Linking Studies on the Interaction of *Escherichia coli* RNA Polymerase with T7 DNA†

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ABSTRACT: We have identified the subunits of *Escherichia coli* RNA polymerase which are in close contact with the T7 phage DNA template using photochemical cross-linking. In nonspecific T7 DNA–enzyme complexes which occur in all regions of the DNA, subunits σ, β, and β' were cross-linked to the DNA. In contrast, in specific binary complexes which presumably occur at promoter sites, and in the initiation complex (holoenzyme + T7 DNA + initiator dinucleotides + three nucleoside triphosphates), only σ and β were cross-linked to DNA, while cross-linking of β' could not be demonstrated. These results (1) do not support the idea that α subunits are involved in the enzyme–template interaction, (2) raise the possibility that σ subunit participates directly in promoter recognition even though isolated σ does not bind to DNA, and (3) indicate different modes of interaction between RNA polymerase and DNA in nonspecific and specific complexes. These findings are relevant to the mechanism by which RNA polymerase carries out selective transcription.

The first step in gene transcription involves the interaction of RNA polymerase with the DNA template. This interaction leads to the formation of a binary complex which is able to bind substrates and initiate RNA synthesis. Two types of binary complexes can be formed: "specific" complexes at discrete sites on the template and "nonspecific" complexes which occur in all regions of the DNA, probably due to electrostatic interactions. In systems where RNA polymerases carry out selective transcription, specific binary complexes have been shown to form primarily at promoter regions of the DNA template, which contain the start signals for correct RNA synthesis (Chamberlin, 1976). The nonspecific binding of RNA polymerase to DNA is thought to play a role in facilitating promoter site selection, the step at which part of the cellular regulation of transcription takes place (von Hippel et al., 1974).

In order to understand the molecular mechanism of transcription, it is essential to know how RNA polymerase selects and recognizes promoter sites on the DNA template. A good system for studying the mechanism of promoter site selection is the transcription of bacteriophage T7 DNA. In vivo and in vitro transcription of T7 DNA by *E. coli* RNA polymerase starts near the 3′ end of the r strand (Summers & Siegel, 1969), where three major promoters have been mapped (Dunn & Studier, 1973; Minkley & Pribnow, 1973). Visualization by electron microscopy has located RNA polymerase on these promoters at positions which agree with those determined by transcription studies (Darlix & Dausee, 1975). Little is known, however, concerning the specific interactions that allow RNA polymerase to distinguish promoter sites from the numerous nonspecific binding sites along the DNA.

In this communication, we describe the spatial relationship of the different subunits of RNA polymerase relative to DNA in both specific and nonspecific enzyme–template complexes. Photochemical cross-linking was used to identify the subunits of the enzyme which are in close contact with the DNA template. This technique has recently been applied to investigate the interaction between *lac* repressor and *lac* operator DNA

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(Lin & Riggs, 1974; Ogata and Gilbert, 1977), and between histones and DNA in chromatin (Martinson et al., 1976).

Experimental Procedures

Preparation of Enzyme and DNA. RNA polymerase holoenzyme was purified from *E. coli* B (Grain Processing Corp.) by the method of Burgess & Jendrisak (1975). The enzyme was 96–98% pure and contained all subunits (α, β, β', and σ) as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[1] For experiments involving specific RNA polymerase–DNA complexes, further purification of holoenzyme ($\alpha_2\beta\beta'\sigma$) was achieved by zone sedimentation in 10–30% glycerol gradients in 0.05 M Tris-HCl (pH 8), 0.5 M NaCl, 10^{-3} M dithiothreitol, and 10^{-4} M EDTA. Core polymerase ($\alpha_2\beta\beta'$) and σ subunit were prepared from holoenzyme by the procedure of Berg et al. (1972). T7 DNA-dependent RNA synthesis by holoenzyme was assayed as described elsewhere (Yarbrough & Wu, 1974).

Tritium-labeled and unlabeled T7 DNA were isolated from a wild-type phage grown in the thymine-requiring *E. coli* strain 011′B (Studier, 1969). Bacteria were grown at 30 °C in the medium of Fraser & Jerrel (1953) containing 2 μg/mL thymidine until $A_{590} \approx 0.5$. For labeled DNA, [*methyl*-^{3}H]thymidine (Schwarz/Mann) was added to obtain a final concentration of 2 μg of thymidine per mL of culture with the specific activity of about 8 μCi/μg. Growth was continued to an A_{590} value of about 1.2 at which point phage infection was carried out at a multiplicity of infection of 0.1–0.2. Infection was allowed to proceed 3–4 h. Following lysis, phage was isolated as described by Studier (1969). DNA was extracted three times with neutralized distilled phenol and then dialyzed to remove traces of phenol. The specific activity of labeled DNA was 100–200 cpm/pmol nucleotide. Analysis by sedimentation in alkaline sucrose gradients (Studier, 1965) revealed that the DNA had less than one break per single strand. The DNA concentration was determined by absorbance using $E_{260}^{1\%} = 200$ (Richardson et al., 1964).

Tritium-labeled poly(dA-dT) was synthesized according to Radding & Kornberg (1962) using DNA polymerase I and [^{3}H]dATP.

Photo-Cross-Linking and Analysis of Photoproducts. Binary enzyme–DNA complexes were formed by incubation of RNA polymerase and T7 DNA in 0.02 M Tris-HCl (pH 8), 0.05 M NaCl, 0.01 M $MgCl_2$, and 10^{-4} M EDTA for 10 min at 37 °C. Ternary initiation complexes were formed by incubating holoenzyme and T7 DNA in the above solution containing 50 μM each ApC, CpA, CpC, CpG, CpU, GpA, GpC, UpA, UpC, and UpG for 10 min at 37 °C. After addition of 50 μM ATP, CTP, and GTP to the reaction mixture, incubation was continued for another 5 min at 37 °C. Initiation was terminated by an eightfold dilution of the sample into Cs_2SO_4 solution to give a final Cs_2SO_4 density of 1.43 g/mL.

Photo-cross-linking reactions were carried out in 0.5 to 16 mL quartz cuvettes with a path length $\leq$1 cm using a Mineralight UVSL-25 lamp operating at 254 nm. The optical density of the solution at 254 nm was kept below 2. Samples were exposed to surface energy densities of 0.5–1.0 J/cm^2, and the exposure time varied from 20 min to 4 h depending on dose and distance from the lamp.

After exposure, samples were treated with nucleases to digest free DNA as described by Giacomoni et al. (1974). This involved a 30-min incubation at 37 °C with 2 μg of pancreatic DNase I (Worthington) per μg of DNA, followed by a 30-min incubation at 37 °C with 0.6 μg snake venom phosphodiesterase (Boehringer or Worthington) per μg of DNA, and finally a 60-min incubation at 37 °C with 0.2 μg of alkaline phosphatase (Boehringer) and 0.6 unit of *Staphylococus aureus* nuclease (Boehringer) per μg of DNA in the presence of 0.01 M $CaCl_2$. For samples photoreacted at high salt concentrations, the solutions were either diluted or dialyzed to lower the ionic strength (<0.1) before nuclease treatment. After degradation of the DNA by nucleases, samples were chilled to 0 °C and precipitated with 10% cold trichloroacetic acid. To photoexposed complexes containing less than 20 μg of enzyme, 10 μg of RNA polymerase was added before the addition of Cl_3CCOOH. The samples were centrifuged at 16 000g for 10 min. The pellets were washed once with 5% cold Cl_3CCOOH and twice with acetone to remove Cl_3CCOOH. The acetone was removed by vacuum evaporation. After addition of the electrophoresis buffer containing 1% sodium dodecyl sulfate, the sample was denatured by heating on a boiling water bath for 90 s.

Electrophoresis of proteins was performed either in phosphate buffer (Shapiro et al., 1967) in 3-mm thick, 5% polyacrylamide slab gels, or in a discontinuous buffer system (Laemmli, 1970) in 1.5-mm thick, 7.5% polyacrylamide slab gels. To determine the radioactivity profile, the gels were cut into slices 4-mm wide and dried in open glass scintillation vials for 2 h at ~140 °C. Subsequently, 0.5 mL of 30% H_2O_2 was added and the tightly sealed vials were incubated for 4 h at 90 °C until the polyacrylamide was completely digested. After cooling, 5 mL of Aquasol (New England Nuclear) was added to each vial. Radioactivity was counted with a Beckman scintillation counter. Electrophoresis of DNA fragments under denaturing conditions was performed in 15% acrylamide gels in Tris–borate–EDTA buffer in the presence of 7 M urea (Gilbert & Maxam, 1973).

Limited proteolysis of RNA polymerase was performed according to Lowe & Malcolm (1976). Trypsin digestion was carried out for 30 min at 37 °C at a trypsin:RNA polymerase ratio of 1:1000 (w/w) in the presence of 0.1 mg/mL heparin. Chymotrypsin was used at a chymotrypsin:total protein (including nucleases) ratio of ~ 1:75 (w/w) and the proteolysis lasted 15 min at room temperature. Both reactions were stopped by adding 0.3 mg/mL phenylmethanesulfonyl fluoride.

All reagents used in these studies were of the best commercial grade available.

Results

Nonspecific Binding of RNA Polymerase to T7 DNA. Nonspecific binary complexes were formed by binding a large excess of RNA polymerase holoenzyme to [^{3}H]T7 DNA (Hinkle & Chamberlin, 1972a). Such complexes were exposed to UV radiation at 254 nm to cross-link DNA and enzyme. It has been shown that a combination of nucleases can degrade T7 DNA bound to RNA polymerase while leaving intact small DNA fragments "protected" by the enzyme, which are 15–40 base pairs long (Giacomoni et al., 1974). By treating the UV-exposed RNA polymerase–[^{3}H]T7 DNA complexes with these nucleases, the protected DNA fragments obtained would include those which had been photo-cross-linked to the enzyme. Furthermore, since these fragments are small, it can be expected that a subunit of RNA polymerase with a DNA fragment attached to it would have a mobility in $NaDodSO_4$ gel electrophoresis similar to that of the unreacted subunit. Analysis of the radioactivity distribution on the gel would then

[1] The abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

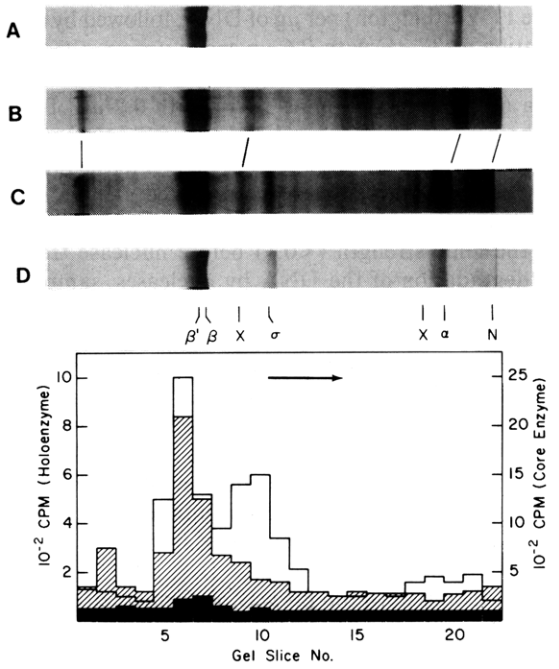

FIGURE 1: Stain and radioactivity profiles of $NaDodSO_4$–polyacrylamide gels of the photo-cross-linked, nonspecific RNA polymerase–[^{3}H]T7 DNA complexes. Holoenzyme (160 μg) and [^{3}H]T7 DNA (8 μg) in 0.4 mL were irradiated at 254 nm with a surface energy density of 0.5 J/cm^2. Core enzyme (85 μg) and [^{3}H]T7 DNA (5 μg) in 0.4 mL were irradiated at 254 nm with a surface energy density of 0.8 J/cm^2. Electrophoresis was performed in discontinuous buffer system in the direction of the arrow. The four gels are (A) unirradiated core enzyme, (B) photo-cross-linked core enzyme, (C) photo-cross-linked holoenzyme, and (D) unirradiated holoenzyme. Stained bands are identified for the corresponding subunits of RNA polymerase. Bands marked "N" are the nucleases used to digest DNA. Bands marked "X" are either the small amount of inter- or intrasubunit cross-linking products or proteolytic products of subunits. In the radioactivity profiles, the open bars represent the photo-cross-linked holoenzyme, the shaded bars represent the photo-cross-linked core enzyme, and the dark bars represent the results of control experiments in which the UV exposure was omitted or 0.5 M NaCl was added to prevent the formation of the binary complex.

allow identification of the subunit photo-cross-linked to ^{3}H-labeled T7 DNA.

Figure 1 shows the gel electrophoresis of the products of photo-cross-linking of nonspecific RNA polymerase–^{3}H-labeled T7 DNA complexes. Following photo-cross-linking with holoenzyme, the radioactivity comigrated with the protein bands corresponding to σ and β and/or β' subunits. Little radioactivity was detected at the position of α subunit. The same pattern indicating involvement of σ and β and/or β' subunits was obtained when RNA polymerase holoenzyme was photo-cross-linked to synthetic poly(dA-dT) (data not shown). Control experiments without UV exposure or experiments in which 0.5 M NaCl was added to prevent formation of the DNA–enzyme complex (Richardson, 1966) revealed no significant radioactivity anywhere on the gel. Photo-cross-linked, nuclease-treated enzyme–DNA complex was isolated by sedimentation in a glycerol gradient in the presence of 0.5 M NaCl at the position of native enzyme. This preparation exhibited the same radioactivity and stain pattern as shown in Figure 1 when analyzed by $NaDodSO_4$ gel electrophoresis. Furthermore, if the photoexposed, nuclease-treated binary complex was treated with proteinase K to degrade the polypeptides, no radioactivity was detected on the gel. These observations indicate that the radioactive DNA fragments were covalently attached to the RNA polymerase subunits by UV irradiation.

It is known that UV radiation causes structural damage to DNA and protein (Wang, 1976; Celis et al., 1976). The photoproducts of DNA are primarily pyrimidine dimers. Using the method of Carrier & Setlow (1971), we estimated that about 5% of the total thymine residues were present as pyrimidine dimers ($\widehat{TT}$ or $\widehat{CT}$) as a result of the radiation doses used in this study. Not to our surprise, the ability of the irradiated T7 DNA to act as template in synthesizing long, trichloroacetic acid precipitable RNA was strongly impaired. However, this DNA was as active as unirradiated DNA in supporting synthesis of pppGpC, the dinucleotide transcribed at the A2 promoter (Oen & Wu, unpublished results). Thus it appeared that the promoter regions of T7 DNA where RNA chain initiation occurred were not significantly damaged by the UV radiation. The polymerization activity of the irradiated enzyme on native T7 DNA template decreased about 50%, even though degradation or aggregation of the protein appeared to be minimal as analyzed by $NaDodSO_4$ gel electrophoresis. To demonstrate that the structural damage of RNA polymerase caused by UV radiation did not significantly alter its subunit contacts with T7 DNA, we have photo-cross-linked RNA polymerase to BrdU-substituted T7 DNA obtained from phage infected *E. coli* 011′B grown in the presence of BrdU (Ogata & Gilbert, 1977). BrdU substitution did not inhibit the nonspecific binding of RNA polymerase to the DNA as determined by nitrocellulose filter assay (Hinkle & Chamberlin, 1972a). Since BrdU possesses significant absorption above 300 nm, irradiation can be performed at a wavelength (316 nm) away from the absorption region of the protein thereby eliminating its radiation damage. We have found that, under these conditions, the RNA polymerase subunits photo-cross-linked to BrdU-substituted T7 DNA were identical with those photo-cross-linked to unsubstituted T7 DNA. Moreover, experiments described below involving specific enzyme–DNA complexes indicate that any UV light induced changes in DNA or protein did not result in the dissociation or reassociation of the binary complexes. Therefore, it seems reasonable that the photo-cross-linking studies described here represent the normal enzyme–DNA interaction.

The gel pattern of the photo-cross-linked core enzyme–^{3}H-labeled T7 DNA complex is also included in Figure 1. The radioactivity comigrated with β and/or β' subunits and no radioactivity was found in the α or σ subunit regions. Thus, the interaction of core enzyme with T7 DNA is similar to that of holoenzyme except for the involvement of σ subunit.

Whether β or β' subunit or both cross-linked to T7 DNA was not resolved by the gel patterns shown in Figure 1. Gels showing better β and β' separation were not useful in solving this problem because a subunit carrying a DNA fragment would not have exactly the same electrophoretic mobility as the uncross-linked subunit due to the finite size of the attached DNA fragment. We have eluted the DNA fragment linked to the β/β' subunit from the $NaDodSO_4$ gel. After treatment with proteinase K in the presence of 20 mM EDTA and 0.1% $NaDodSO_4$ to digest the polypeptide completely, the isolated DNA fragment was electrophoresed in a 15% polyacrylamide gel. In this way, the size of the DNA fragment was estimated to be about 35 bases using *E. coli* tRNA as a standard. With the molecular weight of ~10 000, a 35-base DNA fragment attached to β or β' subunit could alter its electrophoretic mobility sufficiently so as to hinder differentiation between these two subunits. Therefore, a novel approach was sought to identify the subunit photo-cross-linked to the DNA.

Under appropriate conditions, limited degradation of the protein by a protease gives rise to polypeptide fragments of discrete sizes. Lowe & Malcolm (1976) reported that at low

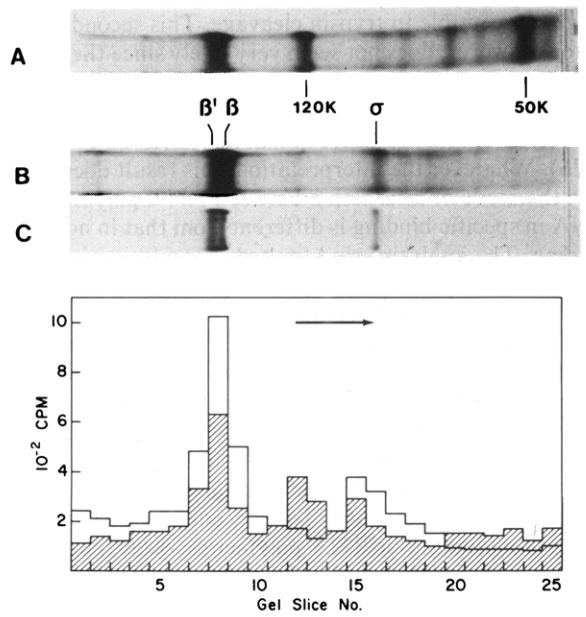

FIGURE 2: Stain and radioactive profiles of $NaDodSO_4$–polyacrylamide gels of the photo-cross-linked, nonspecific holoenzyme–[^{3}H]T7 DNA complexes before and after limited proteolysis with trypsin. Nonspecific binary complexes were formed in 0.2 mL with 80 μg of holoenzyme and 5 μg of [^{3}H]T7 DNA. Photo-cross-linking was carried out by irradiation at 254 nm with a surface energy density of 1.0 J/cm^2. Limited proteolysis with trypsin was as described in Experimental Procedures. $NaDodSO_4$–polyacrylamide gel electrophoresis was in phosphate buffer in the direction indicated by the arrow. Stained bands corresponding to subunits σ, β, β' and two proteolytic fragments of β' subunits with molecular weights 120 000 (120 K) and 50 000 (50 K) are identified in the gel patterns. The α subunit ran off the gel due to long duration of electrophoresis. The three stained gels are (A) the photo-cross-linked holoenzyme–[^{3}H]T7 DNA complex treated with trypsin, (B) the same complex without trypsin treatment, and (C) the holoenzyme marker. The shaded bars shown in the radioactivity profile represent the complex treated with trypsin, whereas the open bars represent untreated complex.

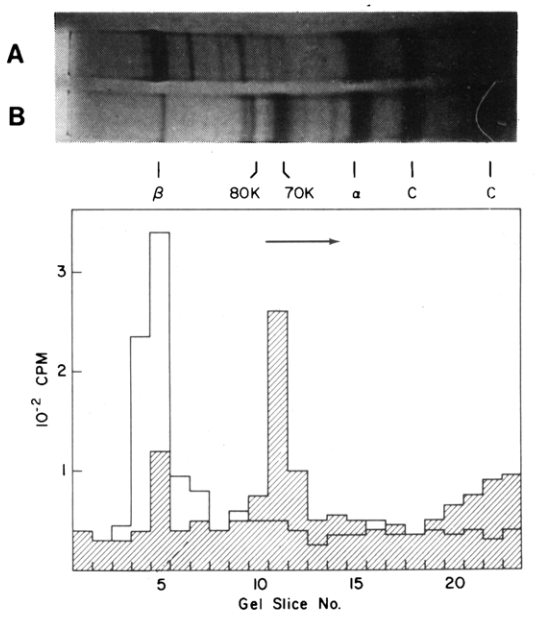

FIGURE 3: Stain and radioactivity profiles of $NaDodSO_4$–polyacrylamide gels of the $\alpha_2\beta$ complexes isolated from the photo-cross-linked nonspecific holoenzyme–[^{3}H]T7 DNA complexes. Nonspecific binary complexes were formed in 2.0 mL with 600 μg of holoenzyme and 30 μg of [^{3}H]T7 DNA and were irradiated at 254 nm with a surface energy density of 0.67 J/cm^2. Isolation of $\alpha_2\beta$ complex and limited proteolysis by chymotrypsin were described in Results and Experimental Procedures. $NaDodSO_4$–polyacrylamide gel electrophoresis was in phosphate buffer in the direction indicated by the arrow. The two stained gels are (A) untreated $\alpha_2\beta$ complex and (B) $\alpha_2\beta$ complex treated with chymotrypsin. Bands corresponding to subunits α and β and two proteolytic fragments of β subunit with molecular weights 70 000 (70 K) and 80 000 (80 K) are identified in the gel patterns. Bands marked C are cytochrome *c* monomer and dimer used to facilitate precipitation of the polymerase subunits. In the radioactivity profiles, the shaded bars represent the $\alpha_2\beta$ complex treated with chymotrypsin and the open bars represent the untreated $\alpha_2\beta$ complex.

ratios of trypsin to RNA polymerase in the presence of single-stranded DNA, the β' subunit in RNA polymerase was cleaved into two polypeptide fragments of approximate molecular weights of 120 000 and 50 000. If the β' subunit were photo-cross-linked to ^{3}H-labeled T7 DNA, one would expect that one or both of the trypsin generated β' fragments would carry radioactivity. The results of such an experiment are shown in Figure 2, where both the large and small β' fragments are seen in the gel of UV-irradiated holoenzyme–^{3}H-labeled T7 DNA complex digested with low levels of trypsin in the presence of heparin. Decreases in radioactivity in the β/β' and σ regions and the appearance of radioactivity in the region of the 120 000 molecular weight fragment were observed for the trypsin-treated complex in contrast to the untreated one. This provides strong evidence that the β' subunit is cross-linked to T7 DNA in nonspecific binary complexes.

A similar approach was followed to ask whether the β subunit was involved in the photo-cross-linking reaction. Chymotrypsin cleaves the isolated β subunit into two fragments of molecular weight about 70 000 and 80 000 (Lowe & Malcolm, 1976). Similar cleavage of β subunit by chymotrypsin can also be obtained with the $\alpha_2\beta$ subunit complex which is an intermediate in the assembly pathway of RNA polymerase. (Isolated β' subunit is also cleaved by chymotrypsin; however, the proteolytic fragments differ in size from those of β subunit.) We have isolated the $\alpha_2\beta$ complex from the photo-cross-linked holoenzyme by zone sedimentation (in a glycerol gradient) of a sample reconstituted at 0 °C from the urea-denatured, photo-cross-linked DNA–enzyme complex (Fukuda & Ishihama, 1974; Palm et al., 1975). When part of the isolated material was subject to $NaDodSO_4$ gel electrophoresis analysis, it revealed mainly α and β subunits in addition to several minor bands. As shown in Figure 3, the radioactivity comigrated with the β subunit exclusively. Gel analysis of the remaining $\alpha_2\beta$ preparation which was treated with chymotrypsin showed the 70 000 and 80 000 molecular weight proteolytic fragments of β subunit. The radioactivity at the position of β subunit decreased significantly and was replaced by a new peak comigrating with the 70 000 molecular weight band. This result indicated the participation of the β subunit in the photochemical cross-linking of holoenzyme to T7 DNA under nonspecific binding conditions.

Specific Binding of RNA Polymerase to T7 DNA. At enzyme levels which do not saturate the available promoters, RNA polymerase holoenzyme forms specific binary complexes at promoter sites on T7 DNA from which the initiation of a specific RNA chain takes place (Hinkle & Chamberlin, 1972a; Le Talaer et al., 1973; Giacomoni et al., 1974). These specific complexes are extremely stable, having a half-time of dissociation longer than 20 h at 37 °C (Hinkle & Chamberlin, 1972b). In contrast, the nonspecific binary complexes which are formed at higher ratios of holoenzyme to T7 DNA dissociate very rapidly (half-times on the order of seconds). Taking advantage of this difference in dissociation rates, we were able to study specific holoenzyme–T7 DNA complexes. Specific and nonspecific holoenzyme–^{3}H-labeled T7 DNA binary complexes were formed at a molar ratio of enzyme:DNA of

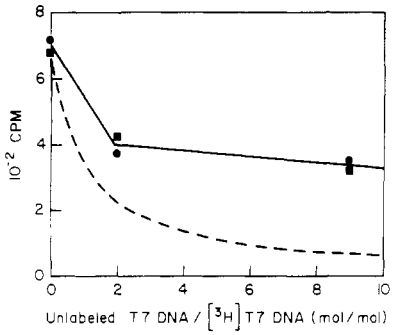

FIGURE 4: Demonstration of specific holoenzyme-[^{3}H]T7 DNA binary complexes by photo-cross-linking. Binary complexes were formed by incubating 20 μg of holoenzyme and 30 μg of [^{3}H]T7 DNA for 5 min at 37 °C. Subsequently unlabeled T7 DNA was added to each sample to give the molar excess relative to [^{3}H]T7 DNA specified on the abscissa. After an additional 25-min incubation at 37 °C, the complexes were irradiated at 37 °C with 0.67 J/cm^2 of UV light. The samples were subjected to NaDodSO$_4$-polyacrylamide gel electrophoresis analysis and the radioactive counts associated with the β and β' subunits were plotted according to the scale shown on the ordinate. The solid line connects the experimental points (two different experiments). The dashed curve represents the theoretical values calculated for photo-cross-linking assuming that all enzyme binding sites on T7 DNA are nonspecific.

33:1 to ensure that all specific binding sites were occupied by active enzyme molecules (there are ~8 tight binding sites for holoenzyme on T7 DNA (Hinkle & Chamberlin, 1972a)). Increasing amounts of unlabeled T7 DNA were then added to remove rapidly dissociating enzyme molecules bound nonspecifically to ^{3}H-labeled T7 DNA (Hinkle & Chamberlin, 1972a). Subsequently these samples were exposed to UV radiation and analyzed by NaDodSO$_4$ gel electrophoresis. In Figure 4, the radioactivity associated with the β and β' subunits is plotted as a function of the amount of unlabeled T7 DNA added to the ^{3}H-labeled T7 DNA–enzyme complexes. After an initial 50% decrease, the photo-cross-linked radioactivity was approximately independent of the amount of competing unlabeled DNA added. The theoretical curve shown in Figure 4 indicates the amount of radioactivity to be detected by photo-cross-linking assuming that all the binding sites are nonspecific and susceptible to competition. Clearly a significant amount of strong (noncompetable) binding has occurred, indicating that roughly one-half the enzyme molecules had formed very stable complexes. Taking into consideration the fact that some fraction of the enzyme may be inactive in transcription (Wu et al., 1975) and, therefore, possibly also inactive in DNA binding as well, the ability of half the enzyme molecules to form stable binary complexes with T7 DNA indicated that there are on the order of ten high affinity binding sites for holoenzyme on T7 DNA. This number is in agreement with a previous estimate (Hinkle & Chamberlin, 1972a). A behavior roughly similar to that shown in Figure 4 was observed for the radioactivity associated with the σ subunit. Thus, β/β' and σ subunits appear to be in close contact with the template in specific binary complexes.

Are both β and β' subunits in close contact with DNA in the specific binary complex? Contrary to the result obtained with the nonspecific complex, no radioactivity was seen to comigrate on the NaDodSO$_4$ gel with either of the two fragments of β' subunit when the photo-cross-linked specific binary complexes were subjected to electrophoresis after proteolysis by trypsin. One interpretation of this result is, of course, that β' subunit is not in close contact with DNA in the specific binary complex. Alternatively, the β' subunit could still be in close contact with DNA in the specific binary complex, but in a conformation different from that in the non-specific complex so that it is no longer susceptible to trypsin cleavage. This second interpretation, however, does not seem very likely since the region of the polypeptide where trypsin cleaves is an easily accessible loop. This is indicated by the observation that four proteases of different specificity cleave in that region (Lowe & Malcolm, 1976). Whatever the interpretation, this result does indicate that the mode of interaction between RNA polymerase and DNA in specific binding is different from that in nonspecific binding. The analysis using limited proteolysis with chymotrypsin revealed that β subunit was photo-cross-linked to T7 DNA in specific binary complexes.

A question may be raised whether the high affinity complexes of RNA polymerase and T7 DNA observed here could be due to the binding of the enzyme to ends or nicks on the DNA (Hinkle et al., 1972). The T7 DNA preparations which were used in the present studies had less than a single break per strand as determined by sedimentation in alkaline sucrose gradients (Studier, 1965). To rule out the possibility that photo-cross-linking of RNA polymerase occurred preferentially at nicks in DNA molecule, we have used ^{3}H-labeled T7 DNA having an average of more than 3 breaks per strand. The results showed that increasing the number of nicks in DNA did not increase the efficiency of photo-cross-linking to RNA polymerase. Another control was performed to determine whether the ends of the DNA molecule were involved in the photo-cross-linking reactions by acting as binding sites for the enzyme. T7 DNA was cleaved with restriction endonuclease MBO I to generate seven DNA fragments (McDonell et al., 1977). A small difference (30% increase) in the radioactivity associated with the enzyme was detected after photo-cross-linking to fragmented as compared with intact T7 DNA preparations, even when the molar ratio of enzyme to DNA was 100. It was concluded that, under these conditions, the DNA ends did not participate significantly in photo-cross-linking to RNA polymerase.

Initiation Complex. The holoenzyme-T7 DNA complex can initiate synthesis of RNA chains in the presence of three nucleoside triphosphates (Naito & Ishihama, 1975) or various combinations of a dinucleotide and one nucleoside triphosphate (Dausse et al., 1975). These initiation complexes are resistant to dissociation by high concentrations of salts such as Cs_2SO_4 or CsCl (Naito & Ishihama, 1975). Using this characteristic salt resistance, we have attempted to determine which subunits of RNA polymerase are involved in DNA binding at this stage of transcription.

Holoenzyme-T7 DNA complexes were initiated in the presence of ATP, GTP, CTP, and a number of dinucleotides. These conditions were used so that initiation could occur at as many promoter sites as possible without allowing synthesis of long RNA chains. Cs_2SO_4 was subsequently added to dissociate the uninitiated complexes. The sample was exposed to UV light and analyzed by NaDodSO$_4$ gel electrophoresis. The gel pattern (shown in Figure 5) revealed photo-cross-linking of β/β' and σ subunits to the DNA. As in the case of specific binary complex, analysis using limited proteolysis with trypsin failed to demonstrate radioactivity in the proteolytic fragments of β' subunit. Again this result suggests that the mode of interaction of RNA polymerase with T7 DNA in the initiation complex is different from that in the nonspecific binary complex possibly because the β' subunit is no longer close enough to cross-link to the DNA.

Discussion

We have performed photochemical cross-linking studies on both nonspecific and specific complexes of *E. coli* RNA polymerase with native, double-stranded T7 DNA. The control

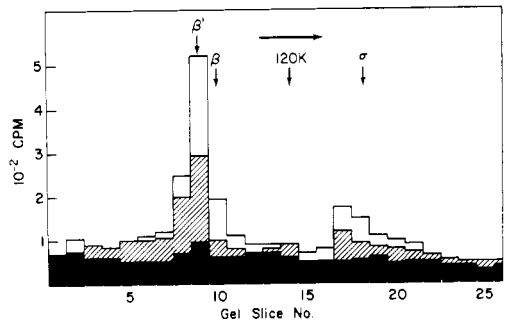

FIGURE 5: Radioactivity profile of $NaDodSO_4$-polyacrylamide gels of photo-cross-linked initiation complexes. The formation of initiation complexes (50 μg of holoenzyme and 65 μg of T7 DNA) and limited proteolysis with trypsin were as described in the Experimental Procedures. Photo-cross-linking was performed by irradiation with 1 J/cm² of UV light. $NaDodSO_4$-polyacrylamide gel electrophoresis was in phosphate buffer in the direction indicated by the arrow. The positions of subunits σ, β, β' and the 120 000 molecular weight (120K) proteolytic fragment of β' subunit on the gel are indicated along the radioactivity profiles. The open bars represent the initiation complexes without proteolysis, and the shaded bars represents the initiation complexes after limited proteolysis with trypsin. The dark bars represent control experiments in which Cs_2SO_4 was added before the nucleotides to prevent the formation of the initiation complexes.

TABLE I: Subunits of *E. coli* RNA Polymerase Holoenzyme Cross-Linked to T7 DNA by UV Radiation.

	subunit			
	α	β	β'	σ
nonspecific enzyme–DNA complex	−	+	+	+
specific enzyme–DNA complex	−	+	−	+
initiation complex	−	+	−	+

experiments have shown that, although some changes in the DNA and enzyme had occurred due to UV irradiation, these left the binding and initiation steps of transcription unaffected. A summary of our results is presented in Table I. Under nonspecific binding conditions, β, β', and σ subunits were cross-linked to T7 DNA indicating that these subunits are in the vicinity of the template in nonspecific binary complexes. In contrast, only β and σ subunits were cross-linked to the DNA in specific holoenzyme–T7 DNA complexes. A finding similar to that for specific binary complexes was obtained for the initiation complex.

Strniste & Smith (1974) were able to demonstrate the induction of stable linkage between *E. coli* RNA polymerase and poly(dA-dT) by UV light. From the density distribution profiles of detergent-treated, UV-induced enzyme–DNA complexes in CsCl equilibrium gradient, they concluded that the cross-linked subunit was most likely σ or α. Frischauf & Scheit (1973) have shown that after photo-cross-linking of core polymerase to ^{32}P-labeled oligo(deoxy-4-thiothymidylic acid), a fraction of the protein-bound radioactivity comigrated with β' subunit on $NaDodSO_4$ gels. Using synthetic poly(dA-dT) as template, we have obtained a cross-linking pattern similar to that of nonspecific holoenzyme–T7 DNA complexes. In addition, the nonspecific interaction of core polymerase with T7 DNA occurs in a manner similar to that of holoenzyme except that σ subunit is not involved. Our finding that three different subunits (β, β', σ) are cross-linked to DNA suggests that a spatially extensive domain of interaction exists between RNA polymerase and the template.

A number of promoter sequences recognized by *E. coli* RNA polymerase are now known (Gilbert, 1976). These sequences exhibit some interesting purine/pyrimidine symmetry, which may reflect the symmetry of the subunit arrangement of RNA polymerase holoenzyme, a concept now widely applied to other protein–nucleic acid interactions (Sobell, 1976). However, the quaternary structure of RNA polymerase holoenzyme as a whole does not contain simple symmetry axes (Hillel & Wu, 1977). The possibility exists that a local symmetry of the enzyme provided by the two α subunits may be involved in the enzyme–template interaction. This possibility is not supported by our result since α subunit was not cross-linked in any of the DNA–enzyme complexes.

Our cross-linking results indicate that β subunit may be in close proximity to the DNA template before and during initiation of transcription. This would seem reasonable in view of the fact that the β subunit possesses the substrate binding sites (Nixon et al., 1972; Frischauf & Scheit, 1973; Wu & Wu, 1974) and, therefore, could also contain the catalytic center of the enzyme. The most surprising finding in our results concerns the β' subunit. This is the only isolated subunit which binds DNA significantly (Sethi & Zillig, 1970; Yarbrough & Hurwitz, 1974). Several mutants of β' have been isolated (Khesin et al., 1969; Panny et al., 1974; Gross et al., 1976) and it has been reported that the mutations resulted in enzyme which either had a lower affinity for DNA (Panny et al., 1974), or was unable to bind to promoter sites (Gross et al., 1976). The most likely interpretation of these cross-linking studies, however, indicates that the β' subunit is involved in the nonspecific, but not the specific interaction with T7 DNA. How can one reconcile these seemingly contradictory observations? One possible role of the nonspecific RNA polymerase-DNA interaction may be to facilitate kinetically the search of the enzyme for promoters. This is reasonable, since there are only a few promoter sites on a very long DNA template and it would be time consuming for RNA polymerase to find a promoter site by random collision or diffusion processes. A kinetically more efficient way would be for the enzyme to bind to one of the multiple nonspecific sites rapidly and then move to the promoter site by some type of site-site transfer mechanism such as a linear diffusion along the DNA molecule (Richter & Eigen, 1974) or a "ring closure" interaction between the protein and two nonadjoining stretches of DNA (von Hippel et al., 1975). It is conceivable that the function of the β' subunit may be to enhance the general affinity of RNA polymerase for the nonspecific sites on DNA. This would facilitate promoter site selection according to the mechanism described above. After the enzyme has reached the promoter site, β' subunit may move away from the DNA template so as to allow β subunit to perform its catalytic function. This, of course, is a hypothetical model which requires further experimental verification. Nevertheless, the difference in our results of photo-cross-linking of β' subunit to DNA in specific and nonspecific enzyme–DNA complexes certainly suggests that the mode of interaction between RNA polymerase and T7 DNA is different for these two types of interaction.

The σ subunit is essential for selection of promoter sites by bacterial RNA polymerase. Hence, the mechanism by which σ acts has been the subject of much interest. This subunit reduces the non-specific binding of core polymerase to DNA by a factor of about 10^4 (Muller, 1971; Hinkle & Chamberlin, 1972a). In addition, it has been pointed out that σ may be involved in the opening of DNA strands by RNA polymerase (Vogt, 1969; Hinkle et al., 1972). In any event, σ could act either directly by binding to DNA (at a promoter site) or indirectly by trapping a specific conformation of core enzyme with an altered affinity for DNA. The possibility of direct binding was considered unlikely (von Hippel & McGhee,

1972) based on the observation that isolated σ does not bind DNA (Zillig et al., 1971). In the present study, we have clearly demonstrated that in the holoenzyme–T7 DNA complexes, σ does "interact" with DNA in a manner which places them in close proximity. In the specific binary complex, such an interaction may be necessary for promoter recognition. At the nonspecific binding sites, the close proximity between σ and DNA could be responsible for reducing the affinity of RNA polymerase for these sites, e.g., by covering part of DNA binding site on core enzyme. The indication by photo-cross-linking of an interaction between σ subunit and T7 DNA in the ternary initiation complex does not come as a surprise since it has been shown that under these conditions σ is not released (Wu et al., 1975). Release of σ from holoenzyme–T7 DNA complexes occurs only in the presence of all four nucleoside triphosphates. Since σ subunit appears to contact the DNA whether the enzyme is bound to a random sequence or a promoter site, it may serve to differentiate between DNA sequences during the binding and initiation steps of transcription. Other possible indirect roles for σ cannot be excluded. Using a fluorescent probe, Wu et al. (1976) have provided kinetic evidence for a conformational transition of RNA polymerase induced by the interaction of σ subunit with core enzyme. Such a conformational change may also explain the altered affinities of σ subunit and core polymerase for DNA after their association to form holoenzyme. Studies on the factors which influence the σ-core interaction during transcription and further studies on the enzyme–DNA interaction with emphasis on DNA strand specificity are currently in progress in our laboratory.

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A Kinetic Study of Cyclic Adenosine 3′:5′-Monophosphate Binding and Mode of Activation of Protein Kinase from *Drosophila melanogaster* Embryos†

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ABSTRACT: Cyclic AMP-dependent protein kinase and its regulatory subunit were isolated from *Drosophila melanogaster* embryos. The profiles of cyclic AMP binding by these proteins were significantly different. In order to explain such a difference and to find the mode of enzyme activation by cyclic AMP, a kinetic study of cyclic AMP binding was carried out. First, the association rate constant k_1 and dissociation rate constant k_{-1} in the cyclic AMP-regulatory subunit interaction at 0 °C were estimated to be 2.3×10^6 M^{-1} s^{-1} and 1.1×10^{-3} s^{-1}, respectively. Secondly, the three possible modes of enzyme activation by cyclic AMP were mathematically considered and could be described by a unique formula: $r = AP^t + BQ^t$ ($A + B = 1$) in which the parameters A, B, P, and Q are equivalent to rate constants in the sense that the rate constants are simply expressed by these parameters. Thirdly, the values of the parameters and subsequently the values of rate constants involved in the possible mechanisms were evaluated using a curve-fitting technique and compared with experimental observation. It was then found that the following mechanism was the only one which fitted the experimental observations. Namely,

$$RC + L \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} LRC \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} RL + C$$

where R, C, and L represent the regulatory and catalytic subunits and cyclic AMP as a ligand. Thus, our results indicate that in the presence of cyclic AMP the active enzyme (C) is released from a ternary intermediate which is the primary product of the cyclic AMP-holoenzyme interaction. The estimated values of the rate constants are: $k_3 = 3.5 \times 10^6$ M^{-1} s^{-1}; $k_{-3} = 7.3 \times 10^{-1}$ s^{-1}; and $k_4 = 3.8 \times 10^{-2}$ s. These estimates indicate that the reaction LRC → RL + C is relatively slow and limits the rate of the overall reaction. By comparing k_{-3} and k_4, it is apparent that a large part of newly formed ternary intermediate reverts to the holoenzyme.

Activity of cAMP-dependent protein kinase (EC 2.7.1.37; ATP: protein phosphotransferase) seems to mediate the effect of cAMP on many eucaryotic processes (for review, see Krebs, 1972; Langan, 1973). From our present knowledge, cAMP-dependent protein kinases, as found in a variety of eucaryotic sources, are all composed of two distinct moieties, i.e., the regulatory (R) and catalytic (C) subunits. This enzyme is therefore a heterodimeric (RC) or heterotetrameric (R_2C_2) holoenzyme (Krebs, 1972; Langan, 1973; Beavo et al., 1975; Rosen and Erlichman, 1975; Hofmann et al., 1975). It is known that cAMP is exclusively bound to the R moiety of a holoenzyme with concomitant release of C, the catalytically active form of the enzyme (Krebs, 1972; Langan, 1973). The mechanism of activation is schematically described as follows:

$$cAMP + RC \rightleftarrows cAMP\text{-}R + C$$

Although the above general paradigm is generally accepted, the precise mode of activation has yet to be demonstrated. Theoretically, a variety of models can be postulated (Ogez and Segel, 1976; Boeynaemes and Dumont, 1977). Furthermore, there exist a number of factors, in addition to cAMP, which are capable of interacting with the enzyme or its subunits and thereby affecting the enzyme activity (Krebs, 1972; Langan, 1973; Haddox et al., 1972; Ashby and Walsh, 1973; Donnelly et al., 1973; Corbin et al., 1973; Rangel-Aldao and Rosen, 1976; Demaille et al., 1977; Szmigielski et al., 1977). It is possible that there exist additional undetermined factors controlling intracellular regulation of protein kinase activity. It is therefore of special importance to learn the mode of activation of the enzyme by cAMP in order to fully understand how the regulation and biological role of protein kinase can involve various interacting factors. In the attempt to study the mode of activation of protein kinase, there are numerous difficulties when the analysis is based upon observations of the relationship between added cAMP and elicited enzyme activity, since the enzyme activation might be considerably affected by reactants such as Mg-ATP (Brostrom et al., 1971; Haddox et al., 1972) and substrate protein (Langan, 1973; Reiman et al., 1971; Miyamoto et al., 1971; Tao, 1972) or where the enzyme itself could be self-phosphorylated and therefore bias activity measurements (Rosen and Erlichman, 1975; Erlichman et al., 1974; Maeno et al., 1974; Hofmann et al., 1975). Observations obtained under such diverse circumstances seem unlikely to permit the recognition of one valid model among the large number of possibilities. Alternatively, several authors have attempted to construct diagnostic models of protein kinase activation based upon binding equilibrium (Ogez and Segel, 1976; Boeynaemes and Dumont, 1977;

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